

A recombinant allergen

This invention relates to a recombinant allergen and in particular to a recombinant Fel d 1, the major cat allergen, which is functionally equivalent to the natural allergen.

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Exposure to airborne particles derived from household cats (*Felis domesticus*) is a common cause of IgE-mediated allergy in, for example, Europe and the US (see Ichikawa, K., et al. (1999) *Clin Exp Allergy* 29, 754-761; Roost, H. P., et al. (1999) *J Allergy Clin Immunol* 104, 941-947 and Lau, S., et al. (2000) *Lancet* 356, 1392-1397). Indeed, cats are found in
10 about 25% of households in Western countries and allergy to cats is found in a large part of the population, e.g. about 10% in the US. The severity of symptoms range from relatively mild rhinitis and conjunctivitis to potentially life-threatening asthmatic exacerbation.

Treatment of cat allergy by allergen injections is often employed but clinical results are
15 variable since for cat allergy, only crude dander extract is available for treatment (see Lilja, G., et al. (1989) *J Allergy Clin Immunol* 83, 37-44 and Hedlin, G., et al. (1991) *J Allergy Clin Immunol* 87, 955-964) although alternative formulations have been proposed (see Norman, P. S., et al. (1996) *Am J Respir Crit Care Med* 154, 1623-1628 and Oldfield, W. L., et al. (2002) *Lancet* 360, 47-53).

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Although patients are occasionally sensitised to several different molecules in cat dander and pelts, e.g. albumin and cystatin, the major allergen is Fel d 1 (i.e. *Felis domesticus* allergen 1, formerly termed Cat 1). The importance of this allergen has been emphasised in numerous studies. In fact more than 80% of cat allergic patients exhibit IgE antibodies to
25 this potent allergen (see Ohman, J. L., Jr., et al. (1977) *J Allergy Clin Immunol* 60, 317-323 and van Ree, R., et al. (1999) *J Allergy Clin Immunol* 104, 1223-1230).

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Fel d 1, was first described 25 years ago as the dominant cat allergen and several subsequent studies have characterised the biochemical and immunological nature of Fel d 1 (see Ohman, J. L., Jr., et al. (1974) *J Immunol* 113, 1668-1677; Leitermann, K., et al. (1984) *J Allergy Clin Immunol* 74, 147-153; Chapman, M. D., et al. (1988) *J Immunol* 140, 812-818; Duffort, O. A., et al. (1991) *Mol Immunol* 28, 301-309; Morgenstern, J. P., et al.

(1991) *Proc Natl Acad Sci U S A* **88**, 9690-9694; van't Hof, W., et al. (1993) *Allergy* **48**, 255-263; van Milligen, F. J et al. (1994) *J Allergy Clin Immunol* **93**, 34-43; Vailes, L. D., et al. (1994) *J Allergy Clin Immunol* **93**, 22-33; Counsell, C. M., et al. (1996) *J Allergy Clin Immunol* **98**, 884-894; Kristensen, A. K., et al. (1997) *Biol Chem* **378**, 899-908 and Batard, T., et al. (2000) *J Allergy Clin Immunol* **106**, 669-676).

The allergen is a 35-39 kDa acidic glycoprotein containing 10-20% N-linked carbohydrates and is found in the pelt, saliva and lachrymal glands of cats. It is formed by two non-covalently linked heterodimers, each consisting of one 70 residue peptide (known as "chain 1") and one 85, 90 or 92 residue peptide (known as "chain 2") encoded by separate genes (see Duffort, O. A., et al. (1991) *Mol Immunol* **28**, 301-309; Kristensen, A. K., et al. (1997) *Biol Chem* **378**, 899-908; Morgenstern, J. P., et al. (1991) *Proc Natl Acad Sci U S A* **88**, 9690-9694 and Griffith, I. J., et al. (1992) *Gene* **113**, 263-268). (Chain 1 shares limited (30%) sequence homology with rabbit uteroglobulin/human Clara cell 10 kDa protein (CC10) and the mature natural (n)Fel d 1 has been associated with gelatin and fibronectin degrading activity.)

Several variants of Fel d 1 have been observed and are reported in the references cited hereinabove. The primary structure of chain 1 is the sequence of SEQ ID NO 1. Reported variants of chain 1 are Lys₂₉→Arg or Asn and Val₃₃→Ser. The primary structure of chain 2 is the sequence of SEQ ID NO 2 or 3. Reported variants of chain 2 are Asn₁₉→Ser, Gly₂₀→Leu, Ile₅₅→Val, Arg₅₇→Lys, Val₅₈→Phe, Glu₆₉→Val, Tyr₇₂→Asp, Gln₇₉→Glu and Asn₈₈→Lys (see Kristensen, A. K, et al. (1997) *Biol Chem* **378**, 899-908).

Furthermore, three inter-chain disulfide bridges linking the two peptides in native Fel d 1 have been identified, i.e. ³Cys(1)-⁷³Cys(2), ⁴⁴Cys(1)-⁴⁸Cys(2) and ⁷⁰Cys(1)-⁷Cys(2), suggesting an anti-parallel orientation of Fel d 1 peptides. See Kristensen, A. K., et al. (1997) *Biol Chem* **378**, 899-908.

Several attempts have been made to associate the separate peptides into a native-like allergen in E coli with only partial success (see Bond, J. F., et al. (1993) *Mol Immunol* **30**, 1529-1541; Keating, K. M., et al. (1995) *Mol Immunol* **32**, 287-293 and Slunt, J. B., et al.

(1995) *J Allergy Clin Immunol* 95, 1221-1228) and recently a soluble and immunoreactive chain 1- linker-chain 2-fusion expressed in baculovirus has been described in which the linker is a peptide having 19 amino acid residues.

5 A mix of the separate chains has proven to be useful for *in vitro* allergy diagnosis, but so far no soluble, stable and correctly folded recombinant (r)Fel d 1 homodimer with retained disulfide formation has been available (see van Ree, R., et al. (1999) *J Allergy Clin Immunol* 104, 1223-1230 and Slunt, J. B., et al. (1995) *J Allergy Clin Immunol* 95, 1221-1228).

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Accordingly, the present invention provides a recombinant Fel d 1 fusion product comprising a Fel d 1 chain 1, a Fel d 1 chain 2 and a linker selected from a carbon-nitrogen bond or a short peptide linker which links the N-terminal amino acid of one chain to the C-terminal amino acid of the other chain.

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The present invention will now be described with reference to the following drawings, in which

Fig. 1 shows the primary structure and disulfide bridges of the recombinant His-tagged rFel d 1(2+1) fusion molecule;

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Fig. 2 shows the results of size exclusion chromatography of rFel d 1(2+1);

Fig. 3 shows the deconvoluted electrospray mass spectrum from analysis of rFel d 1(2+1);

Fig. 4 shows the electrospray mass spectrum from analysis of non-reduced rFel d 1(2+1) digested with trypsin;

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Fig. 5 shows the time-dependent dissociation analysis of immobilised rFel d 1(2+1) by surface plasmon resonance;

Fig. 6 shows the result of a far-UV CD analysis of rFel d 1 and nFel d 1;

Fig. 7 shows the IgE responses to Fel d 1 in individuals sensitised to cats in direct ELISA;

Fig. 8 shows the dose-dependent inhibition of serum IgE from a pool of individuals sensitised to cats; and

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Fig. 9 shows the biologic activity of rFel d 1(2+1) and nFel d 1 in two patients allergic to cats.

The present invention demonstrates that a protein derived from a fusion of the genes coding for the two polypeptide chains constituting Fel d 1 can be folded to resemble closely the structure and allergenic activity of natural (n)Fel d 1. Surprisingly, it has been found that such a recombinant protein mimics the structure and allergenic activity of nFel d 1 when the two peptide chains are linked by a carbon-nitrogen bond or a short peptide linker. Synthetic genes coding for the two Fel d 1 chains may be produced using known techniques, for example by PCR by overlapping oligonucleotides and expression as refolded His-tagged proteins in *E coli*. Two possible constructs, namely one with chain 1 and the other with chain 2 in the N-terminal position, are exemplified. Both constructs have been purified to homogeneity and analysed, in particular for intra-molecular disulfide bridges and homodimer formation using techniques such as size exclusion chromatography, mass spectrometry and surface plasmon resonance.

As explained hereinabove, the two polypeptide chains constituting nFel d 1 are known and described extensively in the prior art. The sequence of chain 1 of nFel d 1 is as shown in SEQ ID NO 1 and the sequence of chain 2 is as shown in SEQ ID NO 2 or 3. However, as well as the variants described hereinabove, any homologous peptide or peptide fragment which has substantially the same sequence and/or provides substantially the same function in the rFel d 1 as chain 1 or chain 2 of nFel d 1 may be used in the present invention. The homologues or fragments of chain 1 or chain 2 must provide substantially the same allergenic activity in the rFel d 1 of the present invention as in nFel d 1. Allergenic activity may be measured by any of the known techniques in the art, such as the response to IgE. Preferably no significant difference in response to IgE between the recombinant and natural Fel d 1 is observed. Preferably the homologues should also provide for the adoption of substantially the same conformation as in nFel d 1.

The linker which links chain 1 and chain 2 is a key feature of the present invention. Although attempts have been made to provide a rFel d 1 using chains 1 and 2 from nFel d 1 with a linker, such efforts have been unsuccessful. The present applicant has found, however, that rFel d 1 which mimics the properties of nFel d 1 may be obtained by linking chain 1 and chain 2 with a linker selected from a carbon-nitrogen bond and a short peptide, i.e. having from 1 to 9, preferably 1 to 5, particularly preferably 1 to 3 amino acids.

Surprisingly, a bond or a short peptide does not induce significant constraints or unfolding, as shown by comparison with nFel d 1 in circular dichroism (CD) measurements.

Preferably the linker is a carbon-nitrogen bond thereby providing a direct fusion between chain 1 and chain 2. An advantage of direct fusion is that no extra amino acids are included within the molecule which otherwise might compromise the use of the molecule in diagnosis and treatment. However, a short (1 to 9 amino acids) peptide bond may be tolerated. An advantage of including a short peptide chain is that the linker may then be used as a target site for a reagent, such as an enzyme, capable of selectively cleaving the polypeptide at the linker. By selective, the applicant means that the polypeptide is cleaved at the linker rather than within chain 1 or chain 2. Such a cleavage would then provide a rFel d 1 having substantially the same structure as nFel d 1, i.e. in which chain 1 and chain 2 are covalently bonded together only by the disulfide bridges.

The present invention therefore also provides a process for preparing a recombinant Fel d 1 peptide comprising the steps of synthesising the peptide having the linker as described herein and selectively cleaving the polypeptide at the linker, using a suitable reagent and preferably an enzyme. Such reagents and enzymes are well known in the art.

The linker links the N-terminal amino acid of one chain to the C-terminal amino acid of the other chain. Preferably the linker links the N-terminal amino acid of chain 1 to the C-terminal amino acid of chain 2 providing a so-called (2+1) construct. Thus, rFel d 1(1+2) contains chain 1 and chain 2 in which the C-terminal of chain 1 is fused to the N-terminal of chain 2 and rFel d 1(2+1) contains chain 1 and chain 2 in which the N-terminal of chain 1 is fused to the C-terminal of chain 2 as shown in Fig. 1 and SEQ ID NO 4. Both chains show specific IgE reactivity although results suggest that rFel d 1(2+1) exhibits superior IgE reactivity to rFel d 1(1+2).

As well as the linker described hereinabove, chains 1 and 2 of the rFel d 1 of the present invention will also be linked by one or more disulfide bridges on account of the presence of cysteine residues in each chain. Biological recognition of proteins is dependent on the primary structures, displayed as linear T cell epitopes in the cavity of MHC molecules on

antigen presenting cells. Equally important for the biological functions are the three-dimensional structures, which in turn depends on secondary structure and frequently on correct and stable disulfide bridges. The rFel d 1 should have at least one disulfide bridge and preferably 2 or 3, particularly preferably 3, as in nFel d 1. The applicant has analysed
5 rFel d 1(2+1) by CD measurements and determined the intra-chain disulfide linking through trypsin cleavage and mass spectrometry. The secondary structure and disulfide bridges pattern of nFel d 1 as well as the proliferation of cultured PBMC in the presence of nFel d 1 were found to correspond well to those observed for rFel d 1(2+1). Thus, the rFel d 1 forms a basis for a stable and immunoreactive allergen

10 An important structural feature of nFel d 1 is the formation of stable non-covalently associated homodimers. The ability of rFel d 1(2+1) to form homodimers has been investigated by several methods, including CD spectroscopy and analysis of IgE antibody responses in direct and competition ELISA using sera from individuals sensitised to cat was
15 carried out. The biological activity was demonstrated by the induction of CD203c on basophils of cat allergic patients.

The 30 kDa rFel d 1(2+1) fraction isolated by size exclusion chromatography (SEC) indicates a homodimer by virtue of its elution position and corresponding molecular
20 weight. The difference in molecular weight to the cat dander derived 35-38 kDa nFel d 1 may be explained by the presence of 10-20% N-linked carbohydrates in the natural allergen. The applicant further investigated the possible homodimer formation via re-chromatography of the isolated 30 kDa fraction by SEC under dissociating conditions. Now the corresponding component eluted as a 15 kDa peak in agreement with the findings from
25 SDS-PAGE using non-reduced sample, suggesting a non-covalently associated dimer. Finally, the rFel d 1(2+1) was analysed by surface plasmon resonance with the assumption that a dissociation rate should be possible to calculate if a dimer was attached to the chip. The time-dependent dissociation indicated a tight protein-protein association which was also supported by the fact that no peak corresponding to the size of a monomer could be
30 detected in SEC. Furthermore, the sensorgram obtained suggested a dimer by the roughly 50% decrease in response measured after deactivation.

From a clinical perspective, as well as for epitope probing, it is important to establish accurate levels of allergen-specific antibodies in serum from e.g. cat-allergic patients. The ability to detect allergen specific IgE in serum from 15 cat-allergic patients to rFel d 1(2+1), nFel d 1 and a mixture of Fel d 1 peptides, was evaluated using direct ELISA. No significant difference in response to IgE was detected for recombinant and natural Fel d 1 indicating that all relevant IgE epitopes are present in the rFel d 1(2+1) structure. Also, the results confirm that the carbohydrate side-chain is not crucial for the folding or else serves as an epitope of nFel d 1. The comparable behaviour of rFel d 1 and nFel d 1 was also implied in the competition ELISA.

rFel d 1 and nFel d 1 revealed the same capacity to compete with IgE in serum for binding to microtitre plate bound nFel d 1 in ELISA. The somewhat better homologous inhibition achieved using high concentrations of nFel d 1 is likely to be caused by the inhibition of antibodies present in the serum pool by matching impurities in the nFel d 1 preparation. A mixture of chains 1 and 2 (with no linker) showed significantly lower IgE binding capacity in direct ELISA and a lower specific activity in the inhibition assay, suggesting a distorted protein preparation with fewer exposed epitopes.

The present invention also provides a DNA sequence encoding the rFel d 1 described herein as well as an expression vector capable of expressing the DNA sequence in an operable form. Although exemplified by *E. coli*, the skilled person would be aware that other known cell lines would be capable of preparing rFel d 1. Accordingly the present invention also provides a host cell transformed with the expression vector as described above. A high level expression *E. coli* system which produces proteins without the attached carbohydrates has been exemplified herein. However, other known systems may be used which provide for the attached carbohydrates and fall within the scope of the present invention.

rFel d 1 of the present invention may be used for diagnosis and therapy of cat-allergic patients. Specifically the present invention provides a pharmaceutical composition comprising an immunotherapeutically effective amount of the fusion product and/or the homodimer as described herein. The present invention also provides for the use of the

fusion product and/or the homodimer as described herein for the preparation of a medicament for the treatment or prevention of cat allergy as well as a method for treating cat allergy, using the fusion product and/or the homodimer as described herein.

- 5 The present invention also provides a kit for the diagnosis of a cat allergy comprising the fusion product and/or the homodimer as described herein as well as a method for the diagnosis of an cat allergy comprising obtaining a sample from a subject and combining with the sample the fusion product and/or the homodimer as described herein.
- 10 Although the subject treated or diagnosed is preferably a human subject, the subject may be any non-human mammal, such a dog.

Examples

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Example 1

Natural and recombinant Fel d 1

- Standard (see Ausubel, F. A., et al. (eds) (1998) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York) or manufacturers' protocols were used for DNA
- 20 manipulations. From published amino acid sequences of Fel d 1 chain 1 and 2 (see Chapman, M. D., et al. (1988) *J Immunol* **140**, 812-818; Duffort, O. A., et al. (1991) *Mol Immunol* **28**, 301-309 and Morgenstern, J. P., et al. (1991) *Proc Natl Acad Sci U S A* **88**, 9690-9694) synthetic genes were made by PCR-amplification (Eppendorf Mastercycler, Hamburg, Germany) using overlapping DNA-primers from DNA Technology A/S (Århus,
 - 25 Denmark) as shown in Table 1 and which are designated as SEQ ID NOS 5-15. In Table 1, restriction enzyme sites (*Nde* I and *Xho* I) are underlined. Forward (F) and reverse (R) primers are indicated.

Table 1

Primer no.	Sequence
1(F)	5'- <u>gtacat</u> atg g aaatctgcc ggctgttaa cgtgacgtg acctgttct gaccggtacc ccggacgaat acgtgaaca ggtg-3'
2(R)	5'-ggcagagctt tgtactgagc aacctgtca acgtattcgt ccgggtgagc aacctgtca acgtattc-3'
3(F)	5'-tgctcagtac aaagctctgc cggttgttct ggaaaacgct cgtatcctga aaaactgcgt tgacgctaaa atgacc-3'

4(R)	5'- <u>cctctcgag</u> g cacagcgggg aggtgtagat ttgtccagc agggacagag cgtttcttt gtctcttcg gtcattttag cgtcaacgc-3'
5(F)	5'- <u>gtacatatg</u> g ttaaaatggc tgaacctgc ccgactctt acgacgtttt ctctgctgtt gtaacggta acgaac-3'
6(R)	5'-ggtagcttc gggtcggtag cgttaactt ggtagggac aggtccagca gcagttcgt accgttagca acagc-3'
7(F)	5'-ctaccgaacc ggaacgtacc gctatgaaaa aaatccagga ctgctacgtt gaaaacggtc tgatctcccg tgtctggac-3'
8(R)	5'-gcttcacca tgtagcttt ggaggaggag atggtgttca taaccagacc gtccagaaca cgggagatca g-3'
9(F)	5'-caaagactgc atgggtgaag ctgttcagaa caccgttga gacctgaac tgaacacct gggtcgtcg agagg-3'
10(R)	5'- <u>cctctcgag</u> a cgaaccaggg tg-3'
11(linker)	5'-cgtttaacag ccgggcagat ttcacgacc aggtgttca gtttc-3'

PCR-reactions (10 µl) containing 1 pmol of each primer, 1-4 for chain 1 and 5-10 for chain 2, using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) was used. The reactions proceeded for 1.0 min at 94°C, 1.5 min at 65°C and 2.0 min at 68°C for 30 cycles. The PCR products were ligated into pT7Blue Blunt Vector, transformed into Nova Blue Single Competent cells using Perfectly Blunt Cloning Kit (Novagen Inc., Madison, WI, USA). Single colonies were grown in 2.5 ml LB medium containing 100 µg/ml ampicillin and plasmids were purified (Qiagen, GmbH, Hilden, Germany) and cut with the restriction enzymes *Nde* I and *Xho* I followed by electrophoretic analysis in 0.3 µg/ml ethidium bromide in 1% agarose gels. Clones with the right size insert (210 and 276 bp for chain 1 and 2, respectively) were sequenced (ABI PRISM® 377 DNA Sequencer, Applied Biosystems). Verified plasmids were used as templates to join chains 2 and 1, using primers 4, 5 and 11 as described for the single chains. After sequencing, the fusion constructs were subcloned into pET 20b (Novagen) using the *Nde* I/*Xho* I restriction sites and transformed into *E. coli* strain BL-21(DE3)pLysS (Novagen). Clones were selected on LB agar plates with ampicillin (100 mg/l) and chloramphenicol (30 mg/l) and grown in LB medium containing 100 µg/ml ampicillin and 30 µg/ml chloramphenicol. pET 20b inserts of chain 1, chain 2 and rFel d 1(2+1) were sequence verified, (ABI). Expression of protein was induced for 3 h in midlog-phase using 0.4 mM isopropyl-thiogalactoside. The cells were pelleted using a J2-21 centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) at 1000xg for 10 min and stored at -20°C. Natural Fel d 1 (>90% pure) from Indoor Biotechnologies (Charlottesville, VA, USA) was used for comparison of immunoreactivity and biological activity between samples.

*Example 2**Purification and characterisation of recombinant Fel d 1*

Protein purification was performed using FPLC, (Amersham Biosciences, Uppsala, Sweden). The same purification protocol was used for all recombinant constructs. The *E coli* pellets containing the expressed recombinant construct were resuspended in 20 mM Tris/HCl, 0.2 M NaCl, 1 mM EDTA, pH 7.4, and disrupted via 8×10 seconds sonication bursts on ice (Soniprep 150 Ultrasonic Disintegrator, Sanyo Gallenkamp, Uxbridge, UK) followed by centrifugation at 12000xg for 25 min (Beckman Instruments). This procedure was repeated twice, after which the pelleted inclusion body preparation was solubilised in 6 M guanidine/HCl, 20 mM Tris/HCl, 0.5 M NaCl, 5 mM imidazole, pH 8.0, and loaded onto a 5 ml Ni⁺-HiTrap affinity column (Amersham Biosciences) operated at 5 ml/min. The column buffer was changed to 6 M urea, 20 mM Tris/HCl, 0.5 M NaCl, 20 mM imidazole, pH 8.0, and a linear gradient was formed to reach 20 mM Tris/HCl, 0.5 M NaCl, 20 mM imidazole, pH 8.0, after 12 column volumes. The protein was eluted with 20 mM Tris/HCl, 0.5 M NaCl, 0.5 M imidazole, pH 8.0. The enriched rFel d 1 preparation was purified by size exclusion chromatography (SEC) (HiLoad® 16/60 Superdex 200 pg, Amersham Biosciences) equilibrated in PBS and in PBS with 0.1% SDS at 1 ml/min. Molecular weight calibration of the column was carried out using bovine serum albumin (BSA), 67kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa and ribonuclease A, 13.7 kDa (Amersham Biosciences) dissolved in PBS or PBS with 0.1% SDS. The relative elution of reference proteins and rFel d 1(2+1) was calculated according to the formula $K_{av} = \frac{V_e - V_o}{V_t - V_o}$ (see Scopes, R. K. (1994) *Protein Purification, Principles and Practice*, 3rd ed. Ed. (Cantor, C. R., Ed.), Springer-Verlag, New York, USA). Purified proteins were stored at -80°C until use.

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The protein concentration of rFel d 1(2+1) was analysed by amino acid analysis using a Biochrom 20 Plus ninhydrin-based analyser (Amersham Biosciences) after hydrolysis at 110°C for 24 h in evacuated tubes with 6 M HCl containing 0.5 % (w/v) phenol. The BCA protein assay (Pierce, Rockford, IL, USA) was sometimes used to estimate the protein concentration. Purity was judged by SDS-PAGE using 15% homogeneous gels and low molecular weight markers (Amersham Biosciences). Samples were denatured at 98°C for 5

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min in SDS sample buffer with or without β -mercaptoethanol (see Ausubel, F. A., et al. (eds). (1998) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York).

Example 3

5 *Electrospray mass spectrometry and determination of disulfide bridges*

For mass determination of rFel d 1(2+1), the folded protein was dissolved at 27 pmol/ μ l in 10 mM ammonium acetate (pH 7.3) and was applied to electrospray ionisation (ESI) mass spectrometry (below) via direct infusion using a syringe pump at 2-5 μ l/min (Harvard Apparatus, Holliston, MA, USA).

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To localise disulfide bridges, 2.7 nmol of the folded rFel d 1(2+1) was dissolved in 10 μ l 9 M urea and incubated 30 min under vortex at 45°C after which 10 μ l water was added. Modified trypsin (5 μ g, Promega) and 10 μ l 0.5 M ammonium bicarbonate (pH 8.0) were added followed by water to yield a final volume of 100 μ l. Digestion proceeded overnight under vortex at 37°C. The reaction was quenched by adding 1 μ l neat trifluoroacetic acid to the sample which was stored at -20°C until analysed. Before mass spectrometry, aliquots of the tryptic digest (10 μ l) were desalted on μ -C₁₈ ZipTips (Millipore, Bedford, MA, USA) and eluted in 60% acetonitrile containing 1% acetic acid for nano-ESI mass spectrometry.

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To make sure that no free sulfhydryl groups existed in rFel d 1(2+1), alkylation was carried out on the non-reduced recombinant preparation (5.4 nmol) using iodoacetamide (Sigma, St. Louis, MO, USA) at 5.5 mM in 20 mM ammonium bicarbonate (pH 8.0) for 15 min at room temperature followed by desalting on μ -C₄ ZipTips and nano-ESI mass spectrometry.

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Mass spectra were recorded using a quadrupole time-of-flight tandem mass spectrometer, Q-TOF (Micromass, Altrincham, UK). The instrument was equipped with an orthogonal sampling ESI-interface (Z-spray, Micromass). Metal-coated nano-ESI needles (Protana, Odense, Denmark) were used and manually opened on the stage of a light microscope to give a spraying orifice of about 5 μ m. This resulted in a flow of approximately 20-50 nL/min when a capillary voltage of 0.8-1.2 kV was applied. A nitrogen counter-current drying gas facilitated desolvation. The cone voltage was set at 40 V.

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The amino acid sequence and the disulfide bridges as determined by nano-ESI mass spectrometry after tryptic digestion of the *E coli* expressed (His)₆-tagged rFel d 1(2+1) fusion protein is shown in Fig. 1 and SEQ ID NO 4. Following column folding and purification by Ni²⁺-chelate chromatography, dominant fractions in SEC with apparent molecular weights of 30 kDa and 51 kDa (Fig. 2) were recovered for further analysis. The 51 kDa component contained inter-chain S-S bonds as shown by a 35 kDa band in non-reducing and a 20 kDa band in reducing SDS-PAGE and was not further analysed (Fig. 2). The 30 kDa fraction, which showed a single 30 kDa peak upon re-chromatography, exhibited 16 and 20 kDa bands by non-reducing and reducing SDS-PAGE respectively, (Fig. 2). When subjected to electrospray mass spectrometry, rFel d 1(2+1) revealed a molecular mass of 19177 Da, which corresponds to the average molecular mass (19183 Da) minus 6 Da, indicating the existence of three disulfide bridges in the structure (Fig. 3). Also seen is an additional peak at 19046 Da, which corresponds to the full-length rFel d 1 without the initiating methionine (Table 1, and residue 0 in Fig. 1 and SEQ ID NO 4). The purity of both the natural and recombinant preparations were >95% as judged by SDS-PAGE (data not shown).

The disulfide bridge formation in rFel d 1(2+1) was analysed by nano-ESI mass spectrometry after trypsin digestion of the non-reduced preparation and the results are shown in Table 2 and Fig. 4.

Table 2.

Tryptic fragment	Residues	Sequence	Theoretical mass [M+H]	Experimental mass [M+H]
T5	44-44	(K)K(I)	147.11	not found
T11	100-100	(K)R(D)	175.12	not found
T14	132-134	(R)ILK(N)	373.28	not found
T1	0-2	(-)MVK(M)	377.22	not found
T4	40-43	(R)TAMK(K)	450.24	450.75
T9	87-92	(K)LNTLGR(E)	673.40	673.40
T16	141-146	(K)MTEEDK(E)	752.31	not found ^a
T3	32-39	(K)VNATEPER(T)	915.45	915.44
T17	147-155	(K)ENALSLLDK(I)	1002.55	not found ^a
T13	122-131	(K)ALPVVLENAR(I)	1081.64	1081/60
T7	58-71	(R)VLDGLVMTTISSSK(D)	1450.78	1450.72
T6	45-57	(K)IQDCYVENGLISR(V)	2156.01	2155.98

T15	135-140	(K)NCVDAK(M)		
T8	72-86	(K)DCMGEAVQNTVEDLK(L)	2408.11	2408.07
T10	93-99	(R)EICPAVK(R)		
T12	101-121	(R)DVDLFLTGTPDEYVEQVAQYK(A)	2430.17	2430.09
T2	3-31	(K)MAETCPIFYDVFFAVANGNELLLDLSLTK(V)	5092.46	5092.28
T18	156-170	(K)IYTSPLCLEHHHHHH(-)		
T16+17	141-155	(K)MTEEDKENALSLLDK(I)	1735.86	1735.80

^a Not found as a separate fragment. Detected as a part of the larger fragment T16 + T17 with [M+H]⁺ 1735.80, resulting from a miscleavage at Lys₁₄₆ which is surrounded by acidic residues, see Fig. 1.

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Example 4

Homodimer dissociation constant analysis

A BIACORE[®]2000 instrument (Biacore AB, Uppsala, Sweden) was employed to investigate homodimer formation of rFel d 1(2+1) by evaluation of the decrease in response relative to maximum binding to the chip surface and the associated dissociation constant. rFel d 1(2+1) and for control purpose a monomer protein, BB (see Batard, T., et al. (2000) *J Allergy Clin Immunol* 106, 669-676), were immobilised onto the surface of CM-5 chips (research grade) via amine coupling to a carboxylated dextran layer using N-hydroxysuccinimide/N-ethyl-N'-(3-diethylaminopropyl)carbodiimide (NHS/EDC) chemistry according to the manufacturers recommendations. The time intervals between surface activation (240-660 s, 35 µl), protein immobilisation (900-1140 s, 20 µl) dissociation phase (1140-1940 s, 67 µl) and surface deactivation (1940-2060 s, 10 µl) were kept constant. In the immobilisation step, 20 µl of a protein solution containing 0.05 µg/µl in 10 mM sodium acetate (pH 4.5) was injected over the NHS/EDC activated surface. After the dissociation phase, the surface was deactivated by injection of 10 µl ethanolamine. The decrease in percent of protein initially attached to the chip surface was calculated as follows: [Response Units (RU) after protein immobilisation at 1230 s – RU after deactivation/RU after protein immobilisation] x 100. All experiments were performed at 25°C and 5 µl/min. The running buffer was 10 mM Hepes (pH 7.4), 0.15 M NaCl, 3.4 mM EDTA, 0.05% surfactant P20. The dissociation constant analysed at 1230-1235 s was based on the equilibrium responses and calculated using the 3.0 software.

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The 30-40 kDa molecular size detected for rFel d 1(2+1) suggests a non-covalent dimerisation similar to that exhibited by natural Fel d 1. This was investigated by BIACORE[®] analysis and by SEC under dissociating elution conditions. In the latter case, the 30 kDa rFel d 1(2+1) fraction produced a single peak corresponding to a molecular weight of 15 kDa using PBS with 0.1% SDS in the running buffer (data not shown). The 30 kDa fraction was further analysed by surface plasmon resonance with the assumption that dissociation of the two subunits can be recorded. As a control, monomeric protein, BB (39), was used, (Fig. 5). The rFel d 1(2+1) construct and the BB monomer bound to the sensor chip in a similar manner. The time-dependent decrease in RU after immobilisation of the rFel d 1(2+1) molecule to the chip surface was 53 %. In contrast, the BB monomer exhibited a stable association to the chip surface during the same time period. In addition, the dissociation constant was determined shortly after the immobilisation phase to be $8.74 \times 10^{-4} \text{ s}^{-1}$.

Example 5

CD measurements

CD measurements of the natural and recombinant Fel d 1 were performed in MilliQ water with protein concentrations of $1.56 \times 10^{-5} \text{ M}$ (here determined using Bio-Rad Protein Assay, Bio-Rad Laboratories, Vienna, Austria). The investigations were carried out on a Jasco J-715 spectropolarimeter (JASCO Labor-und Datentechnik GmbH, Gross-Umstadt, Germany) using a 0.1 cm pathlength cell equilibrated at 20°C. Spectra were recorded with 0.5 nm resolution at a scan speed of 100 nm/min and resulted from averaging 3 scans. The final spectra were baseline-corrected by subtracting the corresponding MilliQ spectra obtained under identical conditions. Results were expressed as the molar mean residue ellipticity [Θ] at a given wavelength. The data were fitted with the secondary structure estimation programs Dicroprot (37) and J-700 (JASCO) using miscellaneous data deconvolution algorithms.

The 20°C CD spectra of natural and recombinant Fel d 1 are nearly identical, characterised by two minima at 208 nm and 222 nm and a characteristic maximum at about 195 nm (Fig. 6). The shape of the spectrum is indicative for a well folded protein with a significant α-helical secondary structure content. The secondary structure estimation resulting from the

fitting procedures yields 35-40 % α -helix and 7-16 % β -sheet structures with root mean square deviations ($CD_{calc} - CD_{exp}$) in the range 4-11 %.

Example 6

5 *ELISA analysis*

Serum specimens from 15 individuals were selected on the basis of positive IgE responses to cat dander (range 0.45-38 kU_A/L using Pharmacia Diagnostics CAP System, Uppsala, Sweden). For control purpose, a pool of 20 non-cat-allergic patients was used. The serum samples were analysed in duplicates by ELISA for IgE antibody binding to rFel d 1(2+1),
10 nFel d 1 or a mix of rFel d 1 chain 1 and chain 2. The assay was performed as a sequential, solid phase adsorption of allergens, serum sample, primary antibody, antibody conjugate and finally substrate including rinsing four times with 250 μ l PBS containing 0.05% Tween 20 (PBS-T) between incubations. If nothing else is stated, all steps were performed at room temperature. Micro titre plates (96 wells, Nunc, Roskilde, Denmark) were coated with 100
15 μ l of rFel d 1(2+1) solution and for comparison also nFel d 1 and an equimolar mixture of chains 1 and 2, to final concentrations of 5 μ g/ml in 0.1 M carbonate buffer, pH 9.6. After over-night adsorption at +4°C, the plates were emptied and the remaining protein binding sites were blocked with 200 μ l PBS-T containing 1% BSA for 2.5 h at room temperature (20-22°C). Each serum sample (100 μ l) was diluted 1:1 in PBS (duplicates) and incubated
20 for 2 h at room temperature, after which 100 μ l rabbit anti-human IgE (Miab, Uppsala, Sweden, diluted 1:2000 (v/v)) was added and incubation was continued for 2 h. Finally, 100 μ l goat anti-rabbit, (Dako, Denmark; diluted 1:2000 (v/v)) conjugated to alkaline phosphatase was added and incubation continued for 1h. Alkaline phosphatase substrate tablets (Sigma 104® Diagnostics, St Louis, MO, USA) were used and the color reaction
25 monitored at 405 nm was registered in an automated ELISA reader (Multiskan RC, Labsystems, Helsinki, Finland). Competition assay of serum IgE was performed using pooled sera from individuals sensitised to cat with more than 10 kU_A/L response to cat dander (mean concentration, 23 kU_A/L), (Pharmacia Diagnostics CAP System). Micro-titre plates (96 wells) were coated with 100 μ l, 5 μ g/ml nFel d 1. Three-fold serial dilutions in
30 PBS-T of rFel d 1(2+1), nFel d 1 and an equimolar mixture of chain 1 and 2 were incubated at a 1:1 volume ratio with the serum pool diluted 1:2 (v/v) in PBS for 2 h at room

temperature and thereafter added to the wells. The subsequent steps were as described for the direct ELISA.

The diagnostic relevance of a recombinant allergen lies in its ability to bind specifically IgE antibodies in body fluids or in tissues from allergic patients in a manner similar to the natural counterpart. This ability of IgE antibodies in sera from 15 subjects sensitised to cat was compared to detect rFel d 1(2+1), nFel d 1 and a Fel d 1 peptide mixture using ELISA. All sera from cat allergic patients showed elevated IgE concentrations compared to a pool of serum from non-cat allergic patients (Fig. 7). Similar responses for rFel d 1(2+1) (optical density (OD) mean, 0.412) and nFel d 1 (OD mean, 0.384) were observed. There was a significantly lower IgE response to the peptide mixture (OD mean, 0.288) compared to rFel d 1(2+1) and nFel d 1, (ANOVA $p < 0.001$ and $p < 0.01$, respectively).

The capacity of serially diluted rFel d 1(2+1), nFel d 1 and a mixture of chain 1 and chain 2 to compete with the binding of patient IgE to microtitre plate bound nFel d 1 was compared using ELISA. All three Fel d 1 preparations exhibited competing activity. The rFel d 1(2+1)-fusion protein inhibited IgE similar as nFel d 1, shown by the proximity and slopes of the dose-dependent inhibition curves in the sensitive range (0.01-0.33 µg/ml), (Fig. 8). The mixture of chain 1 and 2 exhibited more than a 25-fold reduced capacity to compete with IgE binding. By homologous inhibition of nFel d 1, a residual capacity to block IgE was evident using 1 and 3 µg/ml.

Example 7

CD203c assay

The expression of CD203c was performed as described (see Hauswirth, A. W., et al. (2002) *J Allergy Clin Immunol* 110, 102-109). Briefly, heparinised blood samples were taken from two cat allergic patients. Blood aliquots (100 µl) were incubated with dilutions of recombinant and natural Fel d 1 (1 and 10 µg/ml), anti-IgE antibody (1 µg/ml) or PBS for 15 min (37°C). After incubation, cells were washed in PBS/EDTA and then incubated with 10 µl of phycoerythrin-labeled CD203c mAb 97A6 (Immunotech, Marseille, France) for 15 min at room temperature. Thereafter, samples were subjected to erythrocyte lysis with 2 ml

FACS™ Lysing solution (Becton Dickinson, San Diego, CA, USA). Cells were then washed, resuspended in PBS, and analysed by two-colour flow cytometry on a FACScan (Becton Dickinson).

- 5 The biological activity of rFel d 1(2+1) and nFel d 1 was evaluated in cell preparations donated by two cat allergic patients. The surface marker CD203c is upregulated exclusively on basophils in response to allergen cross-linking of the high affinity IgE receptor, FcεRI (38). The capacity of rFel d 1(2+1) and nFel d 1 to activate expression of CD203c on basophils was similar and compared well to that of anti-IgE, which was used as a positive
10 control (Fig. 9a).

Example 8

Lymphoproliferation assay

- Peripheral blood mononuclear cells (PBMC) were isolated from cat-allergic patients by
15 Ficoll (Amersham Biosciences) density gradient centrifugation. PBMC (2×10^5) were cultured in triplicates in 96-well Nunclone plates (Nunc) in 200 µl serum-free Ultra Culture medium (BioWhittaker, Rockland, ME) supplemented with 2 mM L-glutamin (Sigma), 50 µM β-mercaptoethanol (Sigma) and 0.1 mg gentamicin per ml (Sigma) at 37°C and 5% CO₂ in a humidified atmosphere. Cells were stimulated with different concentrations (5,
20 2.5, 1.25 and 0.6 µg per well) of rFel d 1, nFel d 1 and for control purpose with recombinant birch pollen allergen Bet v 1, 4 U Interleukin-2 (IL-2) per well (Boehringer Mannheim, Germany) and medium alone. After 6 days of culture, 0.5 µCi per well [³H]thymidine (Amersham Biosciences) was added and 16 h thereafter incorporated radioactivity was measured by liquid scintillation counting using a Microbeta scintillation
25 counter (Wallac ADL, Freiburg, Germany) and mean counts per minute (cpm) were calculated from the triplicates. The stimulation index (SI) was calculated as the quotient of the cpm obtained by antigen or interleukin-2 stimulation and the unstimulated control.

- The lymphoproliferative responses after challenge of cultured PBMCs with rFel d 1(2+1)
30 and nFel d 1 were analysed by cell incorporation of [³H]thymidine. Both rFel d 1(2+1) and nFel d 1 exhibited equally good proliferation in contrast to the major birch pollen allergen

Bet v 1, which was used as a negative control (Fig. 9b). The T cell proliferation inducing growth factor IL-2 was used as a positive control.

Example 9

5 *Statistical analysis*

Serological results using rFel d 1(2+1), nFel d 1 and the Fel d 1 peptide mixture in the direct ELISA were compared employing ANOVA repeated measures. A p-value <0.05 was considered statistically significant.